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Original Article

MicroRNA-300 plays as oncogene by promoting proliferation and reducing apoptosis of liver cancer cells by targeting MDC1

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Abstract: Increasing reports suggested that deregulated microRNAs (miRNAs) might provide novel therapeutic targets for cancers. However, the expression and function of miR-300 in hepatocellular carcinoma (HCC) was still unknown. In our study, we found that the expression of miR-300 was upregulated in HCC tissues and cell lines compared with paired adjacent non-tumor tissues and HCC cells using qRT-PCR. Further, gain- and loss-of-function studies indicated that transfected with pMSCV-miR-300 greatly promoted cell proliferation and reduced cell apoptosis, while opposite results were found transfected with the miR-16 inhibitor. Moreover, we identified that mediator of DNA damage checkpoint protein 1 (MDC1), a new tumor suppressor gene, was a direct target of miR-300 by luciferase assay. In addition, we found that miR-300 suppressed the expression of MDC1 in HCC cells. Therefore, our results identify an important role for miR-300 in HCC through regulating MDC1 expression.

Keywords: miR-300, mediator of DNA damage checkpoint protein 1 (MDC1), HCC cells

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death and the fifth most commonly diagnosed malignancy worldwide [1]. Hepatitis B and C viral infections, aflatoxin B1, and alcohol are major environmental causes of HCC, but there is no general treatment for HCC [1]. In nowadays, therapeutic options for HCC are limited in their efficacy and specificity. Therefore, it is crucial to identify novel molecules and novel alternative therapeutic strategies to improve clinical outcome of patients suffering from HCC.

Small non-coding RNAs consisting of 21~25 nucleotides called microRNAs (miRNAs) induce mRNA degradation or suppress mRNA translation by binding to the 3'-untranslated region (3'-UTR) of target mRNAs [2-7]. It has been demonstrated that miRNAs play crucial roles in cell biology such as cell proliferation, apoptosis, cell cycle, migration and invasion [8-14]. Increasing evidences had suggested that miRNAs were deregulated or upregulated in all types of cancers, acting either as tumor suppressors or oncogenes according to their target genes [15-18]. In previous studies, researchers indicated that the expression levels of miR-300 was upregulated in several cancers, including gastric cancer [19], breast cancer [20], Osteosarcoma [21] and etc, which suggested that miR-300 functions as an oncogene in tumorigenesis.

In this study, we showed the expression of miR-300 was increased in HCC tissues compared with paired adjacent nontumor tissues and that ectopic expression of miR-300 promoted HCC cell proliferation and reduced apoptosis in vitro. Moreover, MDC1 was identified as the direct functional target of miR-300 in HCC.

Materials and methods

Tissue samples and cell lines

A total of 20 HCC and 20 non-cancerous liver tissue samples were obtained from Affiliated 1st Hospital of Dalian Medical University (Dalian, China). Written informed consent forms

were obtained from all subjects according to the Declaration of Helsinki, and the study was approved by the Ethics Committee of Dalian Medical University (Dalian, China) and complied with the Declaration of Helsinki. All the samples were immediately snap frozen in liquid nitrogen after surgery and stored at -80°C before use.

SK-Hep-1, HepG2 and Huh7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were incubated at 37°C in a humidified incubator under 5% CO_2 condition.

Extraction of RNA and quantitative reverse transcription polymerase chain reaction

Total RNA was extracted using the TRIzoL reagent (Applied Biosystems, Foster City, CA). cDNA was synthesized using Reverse Transcription Kit (Applied Biosystems). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses for genes were performed with the SYBRGreen PCR Master mix (Applied Biosystems) on an ABI 7900 System (Bio-Rad). GAPDH which acted as an internal control were as followed: forward 5'-CGCGCCCCCGGTTTCTA-3', and reverse 5'-GGCTCGGCTGGCGAC-3'. The expression level of miR-300 was determined by TaqMan miRNA assays (Applied Biosystems, USA) according to the provided protocol, and U6 small nuclear RNA was used to normalize the expression.

Construction and transfection of plasmids

The human pre-miR-300 sequence was amplified from normal human genomic DNA and cloned into pMSCV vector to generate pMSCV-miR-300. Cell transfection was performed using DharmFECT1 (Dharmacon) until a final concentration of 20 nM. Medium was changed after 6 h. After transfected and cultured for 48 h, cells were collected for Western blot and qRT-PCR analyses.

Cell viability assay

Cells were seeded in the 96 well plates 24 h after transfection at a density of 1500 cells per well. The cell viability assay was performed using Cell Counting Kit-8 (CCK8; Dojindo) according to the manufacturer's protocol. The absorbance at 450 nm was measured. Experiments were performed at three times.

Flow cytometry

For apoptosis assay, the cells were cultured in low-serum medium and collected after 48 h transfection. Cells were subsequently stained with AnnexinV-FITC (eBioscience, USA) and PI for 30 min as described by the manufacturer. Apoptosis cells were analyzed by FACS.

Luciferase reporter gene assays

The 3'-UTR of MDC1 containing putative binding site of miR-300 were amplified and subcloned into pGL3 luciferase promoter vector (Promega, Madison, WI, USA). For the luciferase assays, 100 ng pGL3-MDC1-3'UTR vector were co-transfected in cells with 100 nM pMSCV-miR-300 or control regent, together with 20 ng Renilla luciferase vector (Promega, USA) as an internal normalized control. Cells were harvested 48 h after transfection and the luciferase activities were assayed according to the manufacturer's protocol. Transfections were performed in duplicate and repeat in three times.

Western blot

Total protein from tissue samples and cell lysis were prepared by RIPA buffer. Western blot analysis was performed with standard procedure. Proteins were separated by 10% SDS-PAGE and transferred to membranes. After incubating with primary antibodies, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG as secondary antibodies and using ECL methods to blotting. The primary antibodies were as followed: anti-MDC1 (1:1000; Santa Cruz Biotechnology, USA), anti-β-actin (1:2000; Santa Cruz Biotechnology, USA).

Statistical analysis

All analyses in this study were statistically performed using GraphPadPrism 5.0 software. The results are expressed as the mean of 3 SEM and were compared by Student's t-test. Differences were considered significant at $P < 0.05$.

Results

miR-300 expression was upregulated in HCC tissues

To evaluate the expression of miRNA-300 in clinical specimens, qRT-PCR was used to detect

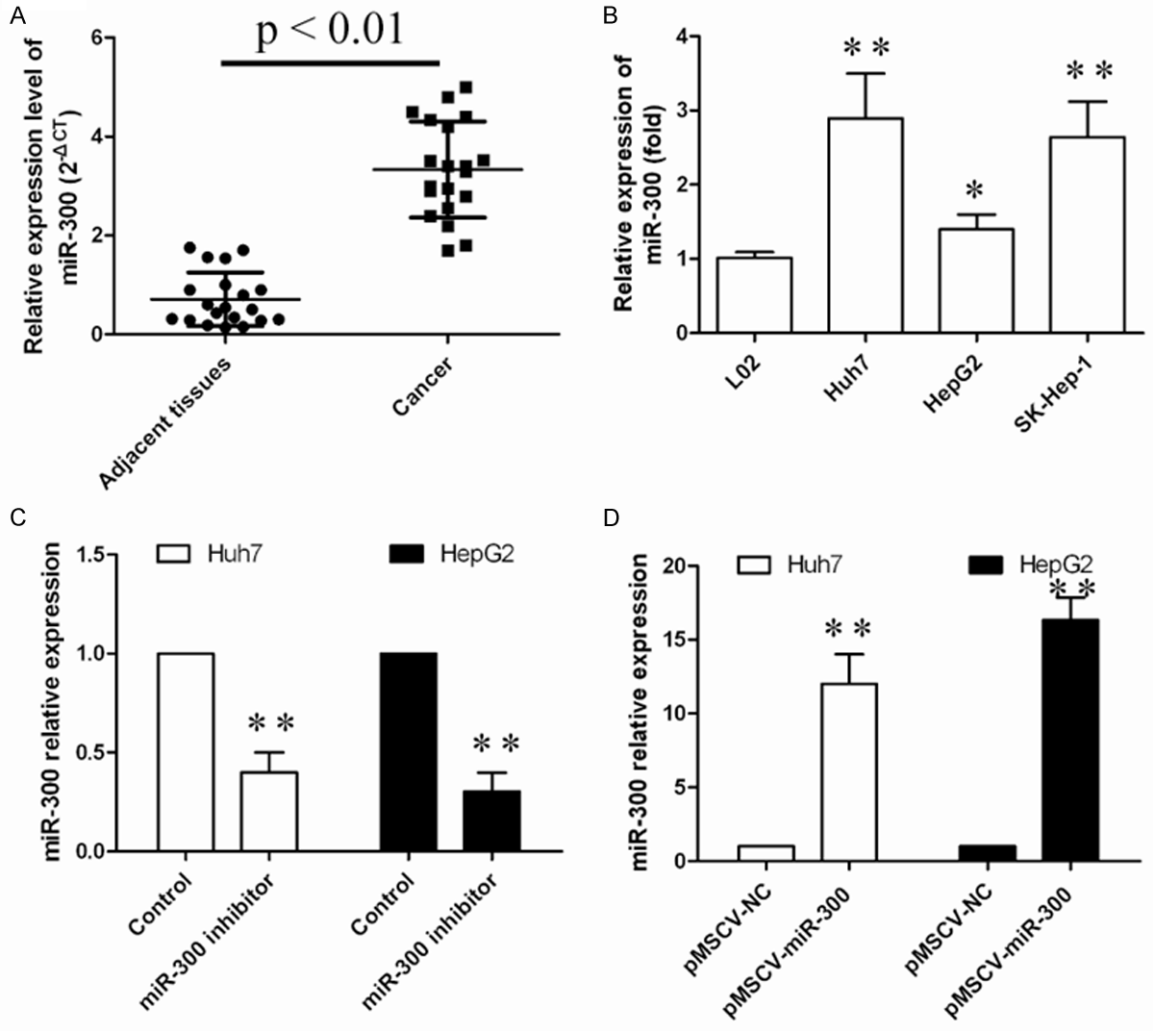

Figure 1. Increased expression of miR-300 in HCC tissues and cell lines. A. Real-time PCR analysis of expression of miR-300 between HCC and matched adjacent normal tissues from HCC patients (n = 20), **P < 0.01. B. Real-time PCR analysis of expression of miR-300 in HCC cell lines (Huh7, HepG2, and SK-Hep-1). The expression of miR-300 was the lowest in HepG2 cells and the highest in Huh7 cells. The data represent the mean ± SD of three different experiments. C and D. Confirmation of overexpression and downregulation of miR-300 in HCC cells using qRT-PCR. The data represent the mean ± SD of three different experiments. *P < 0.05, **P < 0.01.

between 20 pairs of HCC tissues and normal tissue. The results showed that the expression of miR-300 was significantly upregulated in HCC when compared to the nontumorous tissues (**Figure 1A**). It implied that miRNA-300 might be involved in the progression of HCC. Subsequently, we also detected the expression of miRNA-300 in 3 HCC cell lines including Huh7, HepG2, and SK-Hep-1 using qRT-PCR. The human hepatic cell lines LO2 used as a negative control. It was observed that the highest expression of miR-300 was in Huh7 cells and the lowest was observed in HepG2 cells (**Figure 1B**). Given the above results, it was decided to use theHuh7 and HepG2 cells for the below experiments.

To better understand the impact of miR-300 on the HCC cells, the expression of miR-300 was exogenously downregulated in Huh7 cells and the expression of miR-300 was upregulated in HepG2 cells, in which endogenous miR-300 was expressed at a low level or high level respectively, using a lentivirus vector expressing miR-373 (pMSCV-miR-373) or anti-miR-373 inhibitor (**Figure 1C** and **1D**).

miR-300 promoted proliferation and reduced apoptosis of HCC cells

To confirm the impact of miR-300 in HCC cells, we used pMSCV-miR-300 and miR-300 inhibitors to perform the gain and loss function anal-

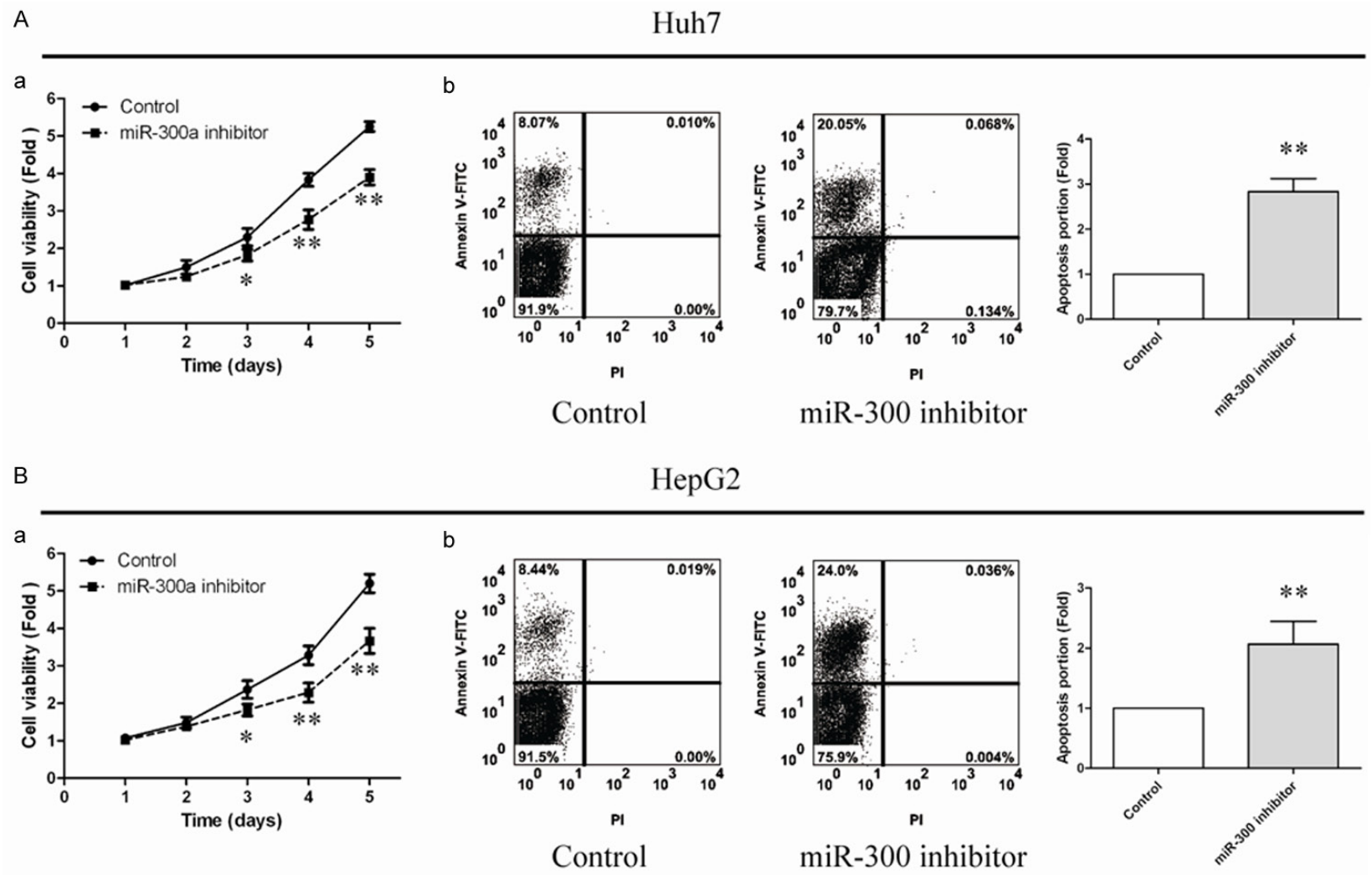

Figure 2. Effects of miR-300 knockdown on cell growth and apoptosis in HCC cells. Aa and Ba. The knockdown of miR-300 resulted in decreased growth rate of the Huh7 and HepG2 cells, (*P < 0.05, **P < 0.01). Ab and Bb. The knockdown of miR-300 promoted apoptosis of the Huh7 and HepG2 cells by flow cytometry method, (*P < 0.05, **P < 0.01).

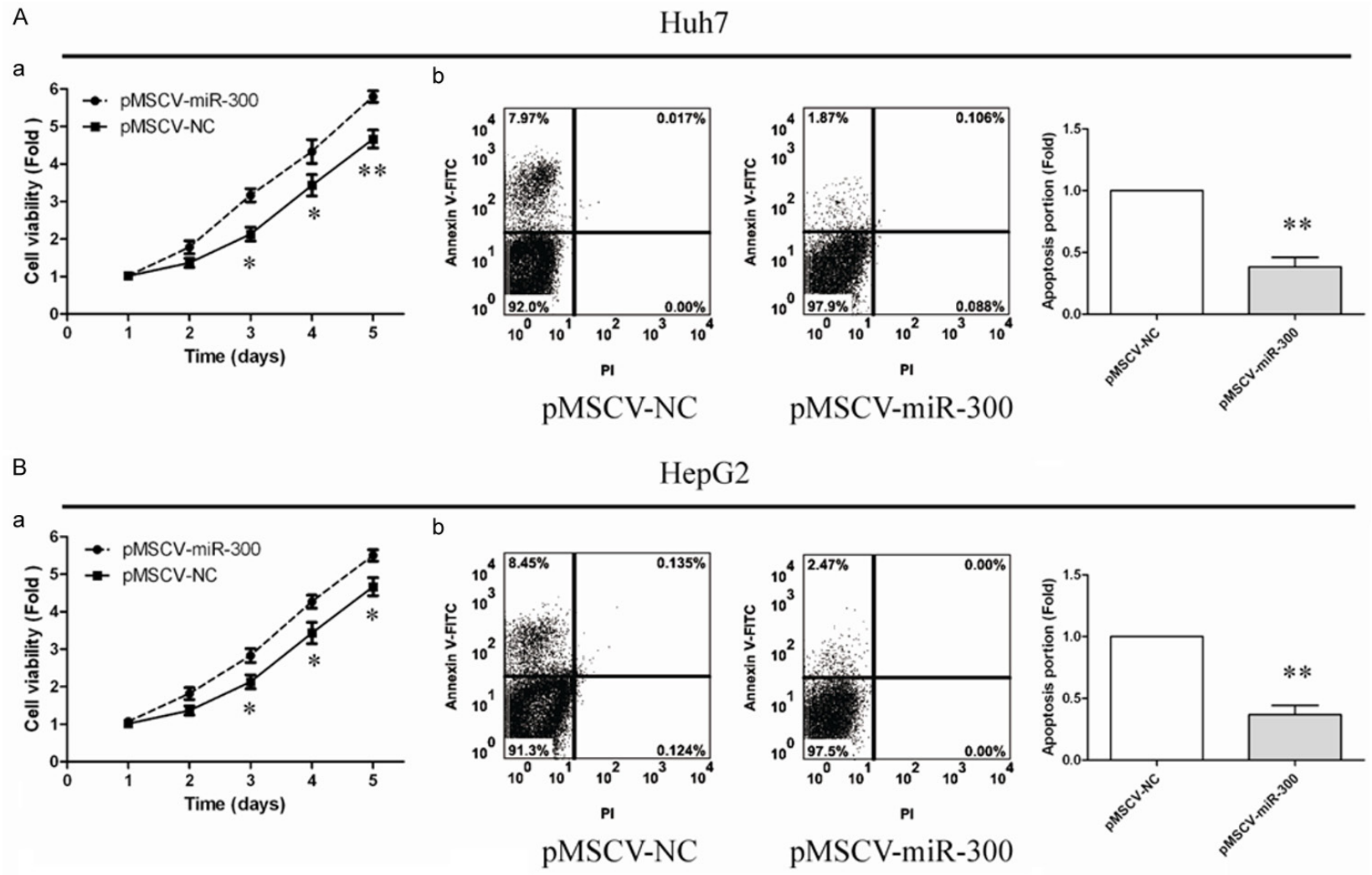

Figure 3. Effects of miR-300 overexpression on cell growth and apoptosis in HCC cells. Aa and Ba. The overexpression of miR-300 resulted in increased growth rate of the Huh7 and HepG2 cells, (*P < 0.05, **P < 0.01). Ab and Bb. The overexpression of miR-300 reduced apoptosis of the Huh7 and HepG2 cells by flow cytometry method, (*P < 0.05, **P < 0.01).

ysis. The results showed that decreased endogenous miR-300 expression suppressed proliferation in HepG2 and Huh7 cells ($P < 0.01$; **Figure 2Aa**, **2Ab**) and increased cell apoptosis (**Figure 2Ba**, **2Bb**). However, inhibited expression of miR-300 could significantly promote proliferation and reduce cell apoptosis of both cells (**Figure 3Aa**, **3Ab**, **3Ba**, **3Bb**). In conclusion, we determined that miR-300 was a positive regulator for HCC progression.

MDC1 was a direct target of miR-300 in HCC cells

To explore the mechanism by which miR-300 affects HCC progression, we employed bioinformatics analysis using Target Scan and attempted to find potential genes which might be involved in miR-300 mediated HCC progression. Previous studies have reported that BRD7 and Twist were two target gene of miR-300 [21, 22]. However, the miR-300 regulation network was still incomplete. In our study, MDC1 was predicted as a potential target gene of miR-300. The predicted binding of miR-300 with MDC1 3'UTR was illustrated (**Figure 4A**). To confirm the direct binding between miR-300 and MDC1 3'UTR, plasmids with MDC13'-UTR or mutated MDC1 3'-UTR were constructed and transfected to HepG2 and Huh7 cells combined with pMSCV-miR-300 or miR-300 inhibitor. The results showed that the luciferase activity increased 4.5-fold in cells expressing pGL3-Wt-MDC1 compared to the pGL3-control in HepG2 and Huh7 cells stably transfected with pMSCV-miR-300 ($P < 0.001$, **Figure 4B**). However, the luciferase activity was not significantly different between cells expressing the pGL3-Mut-MDC1 and pGL3-control in HepG2 and Huh7 cells stably transfected with miR-300 inhibitor (**Figure 4C**). In addition, we also observed that ectopic expression of miR-300 could affect the expression of MDC1 protein in HCC cells (**Figure 4D**). These results indicated that MDC1 was a direct target of miR-300 in HCC cells.

To further elucidate the relationship between miR-300 and MDC1 in HCC, we detected the MDC1 expression in the same 20 paired HCC and adjacent nontumorous tissues. The result showed that the expression levels of MDC1 in tumor tissues inversely correlated with the miR-300 levels (Spearman r: -0.7834, $P < 0.01$; **Figure 4E**). These data suggested that miR-300 negatively regulated MDC1 expression and their inversely correlation could be determined in clinical samples.

Discussion

Dysregulation of miRNAs was detected in various cancers, showing that the dysregulated miRNAs may play a key role in carcinogenesis or tumor progression including HCC. Identifying the miRNAs and their targets that were essential for HCC progression may provide promising therapeutic opportunities. In this study, we found that miR-300 was upregulated in HCC specimens and can promoted HCC cells proliferation and suppressed apoptosis, inferring that miRNA-300 was involved in the development of HCC. Moreover, we demonstrated MDC1 as a direct target of miR-300 and revealed the mechanism of miR-300-mediated MDC1 in metastasis of HCC.

It has been reported that miR-300 could act as an oncogene or a tumor suppressor in different cancers, dependent on cellular context [22, 23]. For example, miR-300 promoted cell proliferation and invasion by targeting BRD7 in Osteosarcoma [21]. Moreover, in breast cancer and gastric cancer, miR-300 might promote cell proliferation and invasion by regulating p53 expression [20]. In this study, we found the expression of miR-300 was higher in HCC tissues and cell lines compared with paired adjacent non-tumor tissues and HCC cells. Also, miR-300 was related to cell proliferation and apoptosis. All the data indicated that miR-300 may play as an oncogene.

MDC1 was a critical component of the DNA damage response (DDR) machinery that participates in DNA damage checkpoint and protects genome integrity [24]. MDC1 was also known as nuclear factor with BRCT (BRCA1 carboxyl-terminal domains) domains protein 1 (NFBDI). Previous evidence indicated that aberrant reduction or lack of MDC1 or 53BP1 in lung and breast cancer by immunohistochemical analysis supported that candidacy of both proteins for tumor suppressors. Recent studies also had demonstrated that loss of MDC1 expression in lung and breast cancer and the increase of tumor incidence in MDC1 knockout mice [24, 25]. Furthermore, MDC1 up-regulation suppressed the process of breast cancer/prostate cancer by enhancing ERα or AR-mediated trans-

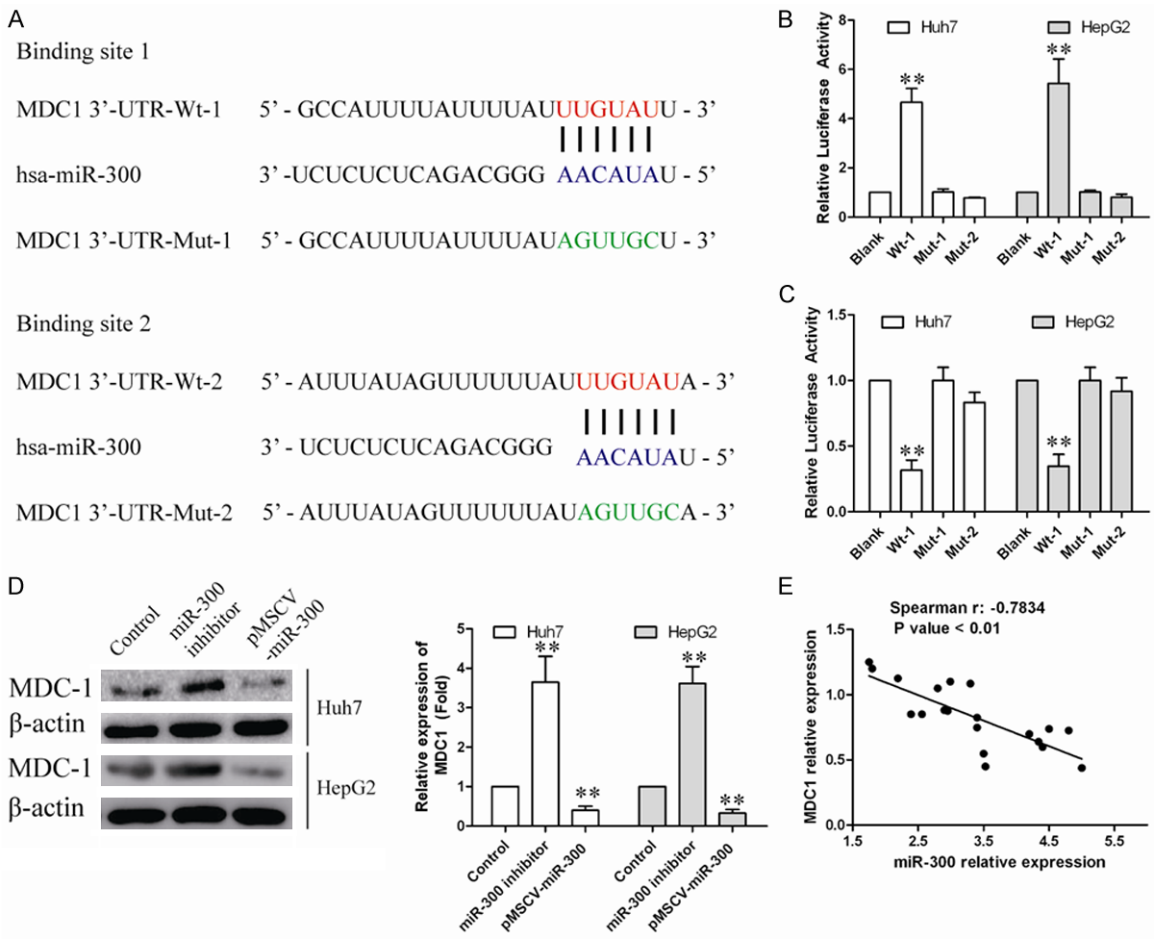

Figure 4. miR-300 targeted MDC1 by binding to its 3'-UTR. A. Bioinformatics prediction between miR-300 and 3'-UTR of MDC1. A predicted seed region in red (3'-UTR-Wt-1 and 3'-UTR-Wt-2) in the 3'-UTR of MDC1 was shown. The mutated sequence (mut-1 and mut-2) used was highlighted in blue (bottom). B. Luciferase activities were measured in Huh7 and HepG2 cells co-transfected with either miR-300 inhibitor or NC oligos and 200 ng plasmid carrying either Wt or Mut 3'-UTR of MDC1, *P < 0.05, **P < 0.01. C. Luciferase activities were measured in Huh7 and HepG2 cells co-transfected with either pMSCV-miR-300 or NC oligos and 200 ng plasmid carrying either Wt or Mut 3'-UTR of MDC1, *P < 0.05, **P < 0.01. D. Western blot analysis showed that ectopic overexpression of miR-300 resulted in decreased MDC1 protein expression in HCC cells. E. miR-300 expression and MDC1 protein level in HCC tissues showed an inverse correlated trend from 20 HCC patients.

activation functions, finally, leading to decreased invasion and migration of breast or prostate cancer [26]. Here, we verified that MDC1 was the target gene of miR-300. MiR-300 directly bound to the 3'-UTR of MDC1, which contained two miR-300-binding sites using a dual-luciferase reporter assay. Up-regulation of miR-300 significantly reduced the MDC1 protein level in HCC cells. Together, these data suggested that miR-300 might inhibit HCC proliferation and apoptosis through regulating MDC1.

In conclusion, the present study demonstrated that miR-300 was increased in HCC tissues and cell lines. Overexpression of miR-300 promoted the cell proliferation and reduced apoptosis of HCC cells through targeting MDC1. To the best of our knowledge, this is the first study to demonstrate that the miR-300/MDC1 axis regulates the proliferation and apoptosis of HCC cells. Repressed miR-300 expression might lead to the increased expression of MDC1 and in turn inhibit the progression of HCC.

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Disclosure of conflict of interest

None.

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